

**Genetic variations in complement factors in patients with congenital thrombotic thrombocytopenic purpura with renal insufficiency**

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**Abstract** The congenital form of thrombotic thrombocytopenic purpura (TTP) is caused by genetic mutations in ADAMTS13. Some, but not all, congenital TTP patients manifest renal insufficiency in addition to microangiopathic hemolysis and thrombocytopenia. We included 32 congenital TTP patients in the present study, which was designed to assess whether congenital TTP patients with renal insufficiency have predisposing mutations in complement regulatory genes, as found in many patients with atypical hemolytic uremic syndrome (aHUS). In 13 patients with severe renal insufficiency, six candidate complement or complement regulatory genes were sequenced and 11 missense mutations were identified. One of these missense mutations, C3:p.K155Q mutation, is a rare mutation located in the macroglobulin-like 2 domain of C3, where other mutations predisposing for aHUS cluster. Several of the common missense mutations identified in our study have been reported to increase disease-risk for aHUS, but were not more common in patients with as compared to those without renal insufficiency. Taken together, our results show that the majority of the congenital TTP patients with renal insufficiency studied do not carry rare genetic mutations in complement or complement regulatory genes.

**Keywords** atypical hemolytic uremic syndrome, complement factors, renal insufficiency, thrombotic thrombocytopenic purpura, Upshaw-Schulman syndrome

## Introduction

Thrombotic thrombocytopenic purpura (TTP), one form of thrombotic microangiopathy, is characterized by microangiopathic hemolytic anemia and thrombocytopenia. The congenital form of TTP, also known as Upshaw-Schulman syndrome, is caused by a severe constitutional deficiency of ADAMTS13 due to homozygous or compound heterozygous *ADAMTS13* mutations [1, 2] and is assumed to represent less than 5% of all TTP cases. The age at disease onset is variable. Some congenital TTP patients present with overt TTP soon after birth, others experience first signs only in adulthood, e.g. triggered by pregnancy, or even remain asymptomatic into their 5th or 6th decades of life [3, 4]. Clinical manifestations in congenital TTP are also heterogeneous. Besides the classical hematological findings, some patients show neurological symptoms, others kidney involvement up to renal failure. Both onset during pregnancy and kidney involvement are noteworthy as they are features also observed in atypical hemolytic uremic syndrome (aHUS), another type of thrombotic microangiopathy [5, 6]. Approximately 60% of aHUS cases can be explained by dysregulation and/or excessive activation of the alternative pathway of the complement system due to mutations in complement regulatory genes (*CFH*, *MCP*, *CFI*, *THBD*), hyperfunctional mutations of complement factors (*C3*, *CFB*) or autoantibodies against complement factor H [5, 6]. Recently, mutations in the diacylglycerol kinase epsilon gene were reported to co-segregate with phenotypic aHUS [7, 8].

To explain the variable clinical phenotypes in congenital TTP, genetic mutations responsible for increased activation of the complement system may influence the severity of renal involvement and thus serve as disease modifiers. Of note in this context is the study of Noris *et al.* who reported on two sisters with congenital TTP who showed different clinical phenotypes [9]. One sister manifested exclusively with neurologic symptoms while the other sister had very severe renal insufficiency that required chronic dialysis. In the latter, a missense mutation in complement factor H was identified which was not present in the sister with neurologic symptoms only. These data suggest that increased activation of the complement system due to genetic mutations modifies the severity of renal involvement in congenital TTP, a scenario derived from a single-family and to be verified in a larger number of patients.

In the present study, we explored whether genetic mutations in complement or complement regulatory genes leading to increased activation of the complement system contribute to the clinical phenotype of congenital TTP patients with predominant renal involvement.

## Patients and methods

### Patients

From two congenital TTP cohorts, that of the Hemostasis Research Laboratory in Bern, Switzerland [10,

11] and the Japanese congenital TTP study [4], confirmed congenital TTP patients were selected based on the following two criteria: a) the patient had not been extensively studied by other groups and b) whole blood for DNA extraction was available. A total of 32 congenital TTP patients (30 from Europe and 2 from Japan) were included in this study, of which thirteen had severe renal insufficiency up to end-stage renal disease (Table 1). The definition for renal involvement was as follows: i) acute renal insufficiency during one or more acute TTP bouts requiring dialysis, or ii) chronic kidney disease defined according to KDIGO (Kidney Disease: Improving Global Outcomes) as persistence of a glomerular filtration rate (GFR)  $<60 \text{ ml/min/1.73m}^2$  or albuminuria for at least 3 months, with or without arterial hypertension; or iii) end stage renal disease requiring renal replacement therapy (either dialysis or kidney transplant); or iv) documented tissue damage on renal biopsy; or v) having a diagnosis of (atypical) hemolytic uremic syndrome established by a nephrologist based on the concomitant presence of thrombocytopenia, microangiopathic hemolytic anemia and renal insufficiency. All patients had severe ADAMTS13 deficiency ( $<10\%$  of the normal) in the absence of a functional inhibitor on at least two time points, at least two ADAMTS13 mutations and/or a plasma infusion trial demonstrating full recovery of infused ADAMTS13 and a plasma half-life of ADAMTS13 of 2-4 days. The plasma ADAMTS13 activity was measured as previously described [12-14]. The study was approved by the Institutional Review Board of each institution.

### Genetic analysis

The coding exons and flanking intronic regions of *CFH*, *C3*, *MCP*, *CFI*, *CFB*, and *THBD* were sequenced as described previously [15, 16] in all 13 congenital TTP patients presenting with renal insufficiency. In the remaining 19 European congenital TTP patients without renal involvement, only a limited analysis of the 11 genetic missense mutations identified in the patients with renal insufficiency was performed. The allele frequencies of the found 11 missense mutations between the 11 European congenital TTP patients with renal involvement and the 19 European congenital TTP patients without renal involvement were compared by chi-square analysis. The nomenclature system of the amino acid and nucleotide numbers is given according to the recommendation of the Human Genome Variation Society. The A of the ATG of the initial Met codon is denoted as nucleotide +1, and the initial Met residue is denoted as amino acid +1. Multiplex ligation-dependent probe amplification analysis was used to screen for deletions of *CFH* and *CFHRs* using a commercially available kit (MLPA kit P236-A2; MRC-Holland, the Netherlands) [15]. The possible impact of the identified genetic mutations on structure and function of the respective proteins was examined by PolyPhen-2 (<http://genetics.bwh.harvard.edu/pph2/>) and SIFT ([http://sift.jcvi.org/www/SIFT\\_enst\\_submit.html](http://sift.jcvi.org/www/SIFT_enst_submit.html)). The crystal structure of the complex of C3b and CCP

1-4 domains of CFH (ID: 2WII) [17] was retrieved from the Protein Data Bank (<http://www.rcsb.org/pdb/home/home.do>). Molecular graphic imaging and analysis were generated using the PyMOL molecular visualization system (Schrödinger, Portland, OR).

In two European patients (Table 1, patient ID 7 and 11), only a single causative *ADAMTS13* mutation had been identified. Therefore, we employed the newly developed genomic quantitative PCR method [18] to identify a second causative mutation.

## Results

To identify genetic mutations in complement genes leading to increased activation of the alternative pathway of the complement system, we performed DNA sequencing of the 6 candidate genes, *CFH*, *C3*, *MCP*, *CFI*, *CFB*, and *THBD* and identified 11 missense mutations in 13 congenital TTP patients with renal insufficiency (Table 1). We retrieved the allele frequency of these missense mutations from population cohorts participating in the NHLBI GO Exome Sequencing Project and the 1000 Genomes project Phase 3 and found that C3:p.K155Q is a rare mutation with a minor allele frequency (MAF) of 0.3% and the two CFB missense mutations, p.L9H and p.G252S, are low frequency mutations with a MAF of 3% (Table 1). These three missense mutations were observed in the European patients. One Japanese patient (ID 13) with renal insufficiency carried the one Japanese-specific CFI missense mutation, p.R201S, which is a low frequency mutation with a MAF of 2% in the Japanese population [19]. The remaining 7 missense mutations were classified as common mutations with a MAF of more than 5%.

We also genotyped the found 10 missense mutations, with exclusion of one Japanese-specific mutation, in the 19 European congenital TTP patients without renal involvement (data not shown). C3:p.K155Q was not identified in this group. None of the remaining 9 missense mutations found in the European congenital TTP patients was significantly more common among patients with compared to those without renal involvement.

Since the *ADAMTS13* mutation, 4143\_4144 insA, is frequent among patients with congenital *ADAMTS13* deficiency in Northern and Central European countries [20], it was frequent in our cohort of European origin. Three of 11 (27.3%) European congenital TTP patients with renal involvement were homozygous carriers for the frequent *ADAMTS13* mutation 4143\_4144insA, as were 6/19 (31.6%) European congenital TTP patients without renal involvement. Though no difference in plasma *ADAMTS13* activity between congenital TTP patients with and without renal involvement was observed, patients without renal involvement were younger (median 33 years; range 14-75 years) than patients with renal involvement (median 43 years, range 29-61 years).

We have recently developed a new genomic quantitative PCR method to identify large gene

deletions in the *ADAMTS13* gene [18]. Employing this method, we identified a second causative mutation in patient ID 7 *ADAMTS13* c.3044+2430\_3568+81del3291, a 3291-bp deletion including exons 24 and 25. In patient ID 11, no large deletion was observed and though the obligatory second mutation still remains unknown, the plasma infusion trial confirmed the congenital TTP diagnosis in this patient.

## Discussion

In the present study, we identified 11 missense mutations in six candidate complement (*C3*, *CFB*) and complement regulatory (*CFH*, *MCP*, *CFI*, *THBD*) genes in 13 congenital TTP patients with renal insufficiency and classified them into rare, low frequency, and common mutations.

The rare missense mutation, C3:p.K155Q found in patient ID 1 is located in the macroglobulin-like (MG) 2 domain of C3, where other mutations predisposing to increased complement activation were previously identified. Figure 1 depicts the location of the C3:p.K155 residue in the crystal structure of the complex of C3b and CFH CCP1-4 domains [17]. The p.K155Q mutation is positioned slightly away from the interface between C3b and CFH CCP1-4, making it unlikely that their interactions are directly affected. Prediction of the impact of this mutation by PolyPhen-2 and SIFT showed “benign” and “tolerated” effects, respectively. For a definite verdict, however, functional studies of the C3:p.K155Q mutation would be needed. In case of common mutation C3:p.R102G, which is also positioned away from the interface between C3b and CFH CCP1-4 (Fig. 1), experimental data indicate that this C3 variant weakly binds to CFH, resulting in reduced CFH cofactor activity thereby favoring alternative complement pathway amplification [21].

The low frequency mutation, CFB:p.L9H within the CFB signal peptide sequence has been reported to be protective for age-related macular degeneration [22]. The low frequency mutation CFB:p.G252S is located in the CFB linker region between the CCP3 domain and the von Willebrand factor A domain. Functional consequences of this mutation are unknown. On the basis of the CFI crystal structure, the low frequency mutation CFI:p.R201S resides on the surface region of the protein away from the proposed cofactor and/or substrate interaction sites, indicating a non-dysfunctional mutation [23]. This mutation is found only in Far East populations including Japanese [19].

The remaining seven mutations found in our cohort are commonly present in the general population. In the study of a rare renal affection, dense deposit disease, both p.R102G and p.P314L mutations in C3 were identified as genetic risk factors for developing this disease [24]. C3:p.R102G is also strongly associated with age-related macular degeneration with an estimated population attributable risk of 22% [25, 26]. In our cohort, both C3:p.R102G and C3:p.P314L were in perfect linkage disequilibrium and five patients with renal insufficiency carried both mutations (Table 1), pointing to

susceptibility for renal involvement through probable hyperactivation of the complement cascade.

Previous functional analyses indicated that the common mutations, CFH:p.V62, C3:p.G102, and CFB:p.R32, are disease-risk mutations [21, 27-29]. The combination of these three mutations yielded 6-fold higher hemolytic activity compared to the protective mutations CFH:p.I62, C3:p.R102, and CFB:p.Q32 [21]. The patient with the rare C3:p.K155Q mutation (ID 1) and two other congenital TTP patients with renal involvement (ID 9 and 11) are carriers of the combined disease-risk mutations (Table 1). The CFH:p.E936D mutation in the CCP16 domain of CFH found in congenital TTP patients ID 2, ID 5, ID 11, ID 12 (in homozygous state) and ID 13 (in homozygous state) has been associated with aHUS [30, 31]. Although these common mutations are not extremely destructive, the combined effects of disease-risk mutations as well as the rare missense mutation C3:p.K155Q may influence susceptibility to renal involvement in congenital TTP patients with hereditary ADAMTS13 deficiency.

Two of 13 congenital TTP patients with renal insufficiency carried homozygous deletions of *CFHR1/CFHR3* genes (Table 1). The complete absence as well as barely detectable levels of CFHR1/CFHR3 are related to the occurrence of autoantibodies to CFH [32] that account for 5-10% of aHUS cases [5]. Therefore, in addition to the above-mentioned missense mutations in complement genes, the deletion of *CFHR1/CFHR3* may contribute to renal affection in congenital TTP patients.

Taken together, our study demonstrates that most of the congenital TTP patients with renal insufficiency do not carry genetic mutations in complement or complement regulatory genes known to predispose to renal insufficiency. Secondly, although some congenital TTP patients with renal insufficiency were found to be carriers of common aHUS-risk mutations, such as CFH:p.V62, CFH:p.D936, C3:p.G102, CFB:p.R32, and homozygous deletions of *CFHR1/CFHR3* genes, these mutations were not more common than in the general population or in our 19 congenital TTP patients without renal involvement.

Microvascular platelet thrombosis and endothelial injury resulting from ADAMTS13 deficiency initiate the coagulation and fibrinolytic pathways, which in turn may contribute to complement activation in congenital TTP [33, 34]. Complement activation with consumption of complement factors has already been demonstrated during acute TTP episodes [34, 35]. Overactivation of the alternative complement pathway also leads to coagulation cascade activation. Our results suggest that rare predisposing complement genetic mutations do not contribute to a large extent to the phenotypic variability in congenital TTP patients. Further, the common aHUS-risk mutations in complement or complement regulatory genes observed in our small series of congenital TTP patients with renal insufficiency were equally frequent in congenital TTP patients without renal failure. Recruitment of larger number of congenital TTP patients with well-defined phenotypes will be necessary to obtain a full

conclusion.

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**Conflict of interest** Dr. Lämmle is a member of the Data Safety Monitoring committee of the BAX 930 Study testing rADAMTS13 in congenital TTP patients. Dr. Fujimura is a recipient of the research fund from Alexion Pharmaceuticals. Other authors have no conflict of interests.

### Figure legend

**Fig. 1.** Location of the C3:p.K155Q and C3:p.R102G mutations in the C3b-CFH CCP1-4 complex. C3b  $\beta$ -chain containing macroglobulin-like domains (MG) 1-5 is shown in blue and C3b  $\alpha'$ -chain is shown in green. Complement control protein (CCP) domains 1-4 in CFH are depicted in orange. A calcium ion is shown as gray sphere. The p.K155 and p.R102 residues in C3b are depicted by magenta spheres. Both mutations, p.K155Q and p.R102G, are not positioned at the contact interface of the two proteins. Diagram was generated with the PyMOL molecular visualization system.



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